Supplemental Information

Mutation of Cullin-3 Causes Arterial Stiffness and Hypertension Through a Vascular Smooth Muscle Mechanism

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Supplemental Figures

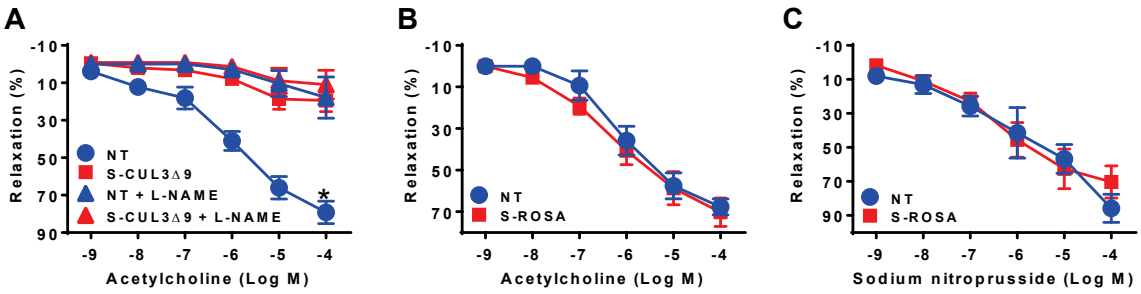

Figure S1: Vascular Function.

A) Effect of the NOS inhibitor, L-NAME (100 µmol/L, 30 min) on ACh relaxation in basilar arteries from NT and S-CUL3Δ9 mice. N=5/genotype or treatment; error bars represent means ± SEM. *p<0.05. vs all other curves. Data were analyzed with two-way repeated measure analysis of variance (ANOVA). B-C) Dose-dependent relaxation of basilar arteries to acetylcholine (B) and to sodium nitroprusside (C) from NT and mice expressing tdTomato reporter specifically in smooth muscle (S-ROSA). N=3-5/genotype.

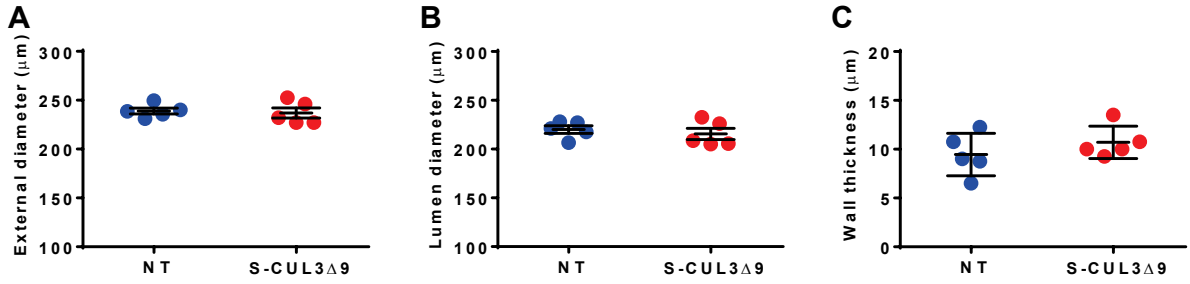

Figure S2: Vascular Structure.

Measurements of external diameter (G), luminal diameter (H) and wall thickness (I) of basilar arteries from NT and S-CUL3Δ9 mice under calcium-free conditions. N=5/genotype.

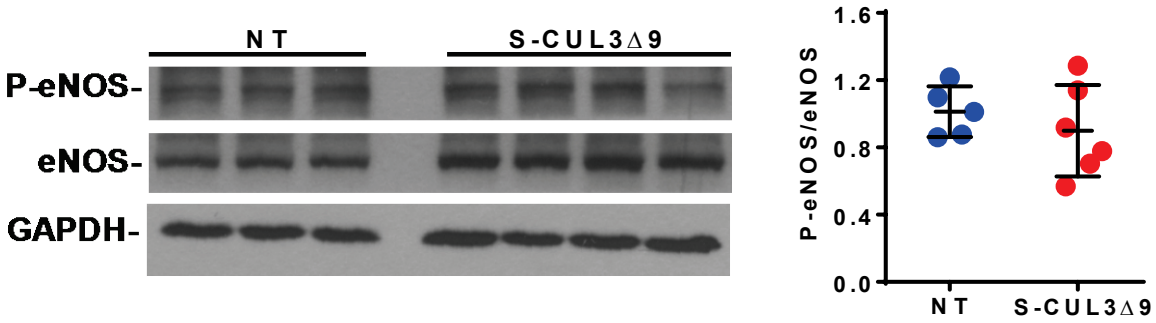

Figure S3: Expression of eNOS in S-CULΔ9 Aorta.

A) Western blot and quantification of phosphorylated and total endothelial nitric oxide synthase enzyme (eNOS) from aorta of NT and S-CUL3Δ9 mice (N=5-6/genotype).

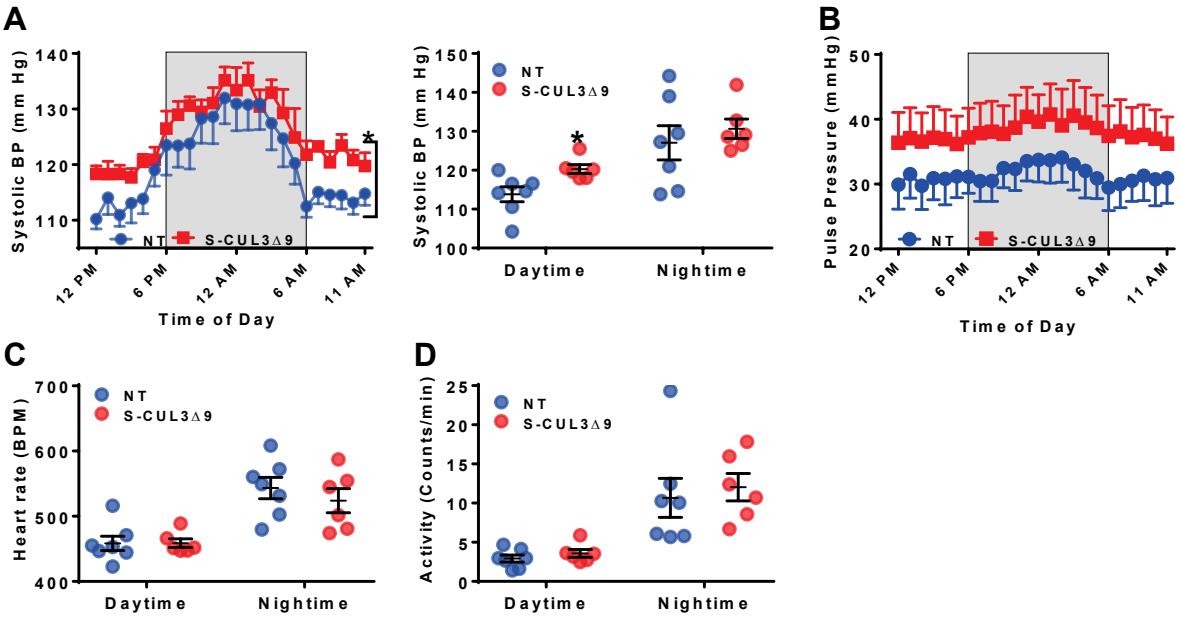

Figure S4: Blood Pressure.

Systolic blood pressure (A), pulse pressure (B), heart rate (C) and activity (D) were measured continuously by radiotelemetry for 7 days 4-weeks post tamoxifen in NT and S-CUL3Δ9 mice. The data are collapsed onto a single 24 hour light:dark cycle. Shaded areas indicate the dark cycle N=6-7/genotype; *p < 0.05 S-CUL3Δ9 vs NT. Data were analyzed with two-way repeated measure analysis of variance (ANOVA).

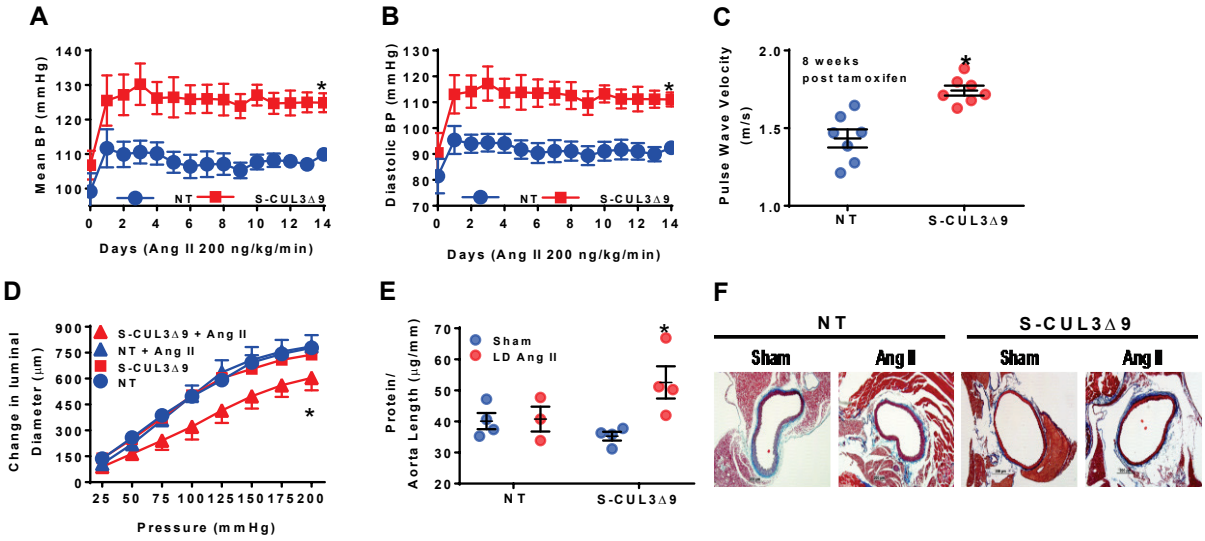

Figure S5: Aortic Compliance.

A-B) Effects of acute Ang-II administration on mean arterial pressure (A) and diastolic blood pressure (B) in NT and S-CUL3Δ9 mice as measured by radiotelemetry. Day 1 indicates the start of Ang-II infusion. N=5/genotype; *p<0.05 S-CUL3Δ9 vs NT. C) Pulse wave velocity measurements in NT and S-CUL3Δ9 mice as determined by Doppler ultrasound 2-months post tamoxifen. N=7/genotype; *p < 0.05 S-CUL3Δ9 vs NT. D) Pressure-luminal diameter curves of NT and S-CUL3Δ9 aorta. N=4-6/genotype or treatment; *p < 0.05 Ang-II S-CUL3Δ9 vs all other curves. Data were analyzed with one-way repeated measure analysis of variance (ANOVA). E) Total protein/aorta length from NT and S-CUL3Δ9 mice. N=3-4/genotype or treatment; *p < 0.05 Ang-II S-CUL3Δ9 vs all other samples. Data were analyzed with one-way ANOVA. F) Adventitial collagen staining as determined by Masson Trichrome in aorta of NT and S-CUL3Δ9 mice.

Supplemental Experimental Methods

Animals: To generate transgenic mice overexpressing CUL3Δ9, we obtain CUL3Δ9 cDNA by splice overlap extension PCR to permanently delete exon 9 of mouse CUL3WTand confirmed by DNA sequencing. CUL3Δ9 cDNA was then cloned into a construct characterized by a CAG promoter (CMV enhancer fused to the ubiquitous chicken β-actin promoter), a lox-STOP-lox sequence, and an IRES-tdTomato reporter for transgene expression. CUL3Δ9 transgenic mice were then generated at the University of Iowa Gene Editing Facility. Next, we generate mice that inducibly express CUL3Δ9 specifically in smooth muscle (S-CUL3Δ9) by crossing CUL3Δ9 transgenic mice with mice expressing a Tamoxifen-inducible Cre-recombinase ($CreERT^2$) under the control of the smooth muscle myosin heavy chain promoter B6.FVB-Tg(Myh11-cre/ERT2)1Soff/J, Jax stock 019079). Tamoxifen was administered (75 mg/kg IP) daily for 5 consecutive days. Age- and sex-matched S-CUL3Δ9 and non-transgenic (NT) control mice were used for the experiments described herein. As additional controls, we generated mice that express the tdTomato reporter in smooth muscle by crossing B6.FVB-Tg(Myh11-cre/ERT2)1Soff/J and the ROSA26 reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J, Jax stock 007914). Tamoxifen (75 mg/kg IP) was administered for 5 consecutive days. Only male mice were used in this study because the BAC transgene was inserted into the Y chromosome of B6.FVB-Tg (Myh11-cre/ERT2)1Soff/J. Mice were maintained under standard housing conditions, with access to food and water ad libitum. All animal studies were approved by the University of Iowa Animal Care and Use Committee and was performed in accordance with the standards set forth by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

PCR Analysis for Recombination: Carotid artery, stomach, liver, brain, kidney, heart and bladder tissues were isolated from NT and S-CUL3Δ9 mice and frozen in liquid nitrogen. Genomic DNA was extracted using a DNeasy blood and tissue kit (Qiagen) following the manufacturer's protocol. We designed primers to amplify a 1300bp band for the intact fragment of the construct or a 430bp band for the recombined fragment where the lox-STOP-lox sequence of the construct was excised. PCR was then performed using Taq DNA polymerase (Invitrogen) following the manufacturer's protocol.

Immunohistochemistry: Aorta from NT and S-CUL3Δ9 mice were dissected free of adventitial fat, embedded in OCT compound and kept frozen at -80°C until processed for sectioning. Frozen aortic sections (10 μm thick) were fixed with 4% paraformaldehyde for 15 min at room temperature. Sections were rinsed with 1X PBS three times for 10 min each. Slides were then permeabilized with 0.2% Triton-X-100 in PBS for 30 mins at 37 ^{o}C. Sections were then blocked with blocking buffer consisting of 10% goat serum, 0.1% Triton-X-100, 1% bovine serum albumin (BSA) in 1X PBS for 1 hour. Sections were incubated with primary antibody against tdTomato (catalog # GTX127897, Gene Tex) diluted 1:100, or CD31 (catalog # 553370, BD Biosciences) diluted 1:50, in blocking buffer overnight at 4^{o}C. Sections were rinsed with 1X PBS three times for 10 min each and were incubated with secondary antibody against tdTomato (anti rabbit-568) and against CD31 (anti rat 488) at a final dilution of 1:2000 for 2 hours. Sections were rinsed with 1X PBS three times for 10 min each. Sections were then mounted on glass slides with mounting medium containing DAPI (Vectashield, Vector Laboratories) and images were visualized using confocal microscopy (Zeiss LSM710).

Blood Pressure Measurement and Acute Ang-II Experiments: Blood pressure was measured by radiotelemetry as previously described (1). Briefly, mice 3-4 months, age- and sex-matched were anesthetized with ketamine:xylazine (87.5mg:12.5mg/kg), the catheter was

placed into the left carotid artery and the transmitter subcutaneously placed along the left flank. Mice were allowed to recover for 7 days, after which systolic, mean and diastolic pressure, heart rate and activity were recorded every 5-minute for 10-second intervals for a total of 7 days. Mice were then administered tamoxifen (75 mg/kg IP) for 5 consecutive days and blood pressure was measured at two and four weeks thereafter. Next, an osmotic minipump (Alzet model 1002) was implanted to infuse Ang-II (200 ng/kg/min) for 14 days. All data were collected and stored using Dataquest ART software.

Wire and Pressurized Myography: Wire myograph studies were done as previously described (2, 3). Briefly, thoracic aorta were dissected free of adventitia fat, cut into 3-4 mm sections in length and suspended in an organ bath containing Krebs buffer (NaCl 118.3 mmol/L, KCl 4.7 mmol/L, CaCl2 2.5 mmol/L, MgSO4 1.2 mmol/L, KH2PO4 1.2 mmol/L, NaHCO3 25 mmol/L, glucose 11 mmol/L) bubbled with 95% O2/5% CO2 and maintained at 37°C. Vessels were connected to a force transducer, and following equilibration at 0.5 g for 45 minutes, precontracted with PGF2α to 45-50% of maximal response to U46619. After a plateau was reached, concentration-response curves were determined for acetycholine (ACh) (0.001–100 µmol/L), sodium nitroprusside (SNP) (0.001–100 µmol/L), DEA NONO (0.001–100 µmol/L). Contractile response to potassium chloride (KCl) (10–100 mmol/L), and endothelin 1 (ET-1) (0.1–100 nmol/L) was determined. For Rho-kinase inhibition, aortic rings were pre-incubated with Y-27632 (1 µmol/L) for 30 min and ACh relaxation was conducted. Data was collected using PowerLab and analyzed using Chart5 software (AD Instruments).

Pressurized myograph studies were done as previously described (4, 5). Briefly, basilar arteries were isolated, cleaned of connective tissues and cannulated onto glass pipettes filled with aerated Krebs buffer in an organ chamber. Arteries were then transferred to a DMT pressurized myograph system, equilibrated for 30 min at 60 mm Hg under no-flow conditions. Vessel viability was then determined by KCl (100 mmol/l). Arteries were precontracted with the thromboxane A2 mimetic (U46619) to 30% internal diameter and after a stable precontraction, concentration-response curves were determined for ACh (0.001–100 µmol/L), SNP (0.001–100 µmol/l), angiotensin 1-7 (0.001–10 µmol/L), KCl (6-15 mmol/L), cromakalim (1-10 µmol/L) and nifedipine (0.001–10 µmol/L). Maximum passive diameter was determined at the end of the experiment by exposing basilar arteries from NT and S-CUL3Δ9 mice to Ca^{2+}-free Krebs buffer containing 2 mmol/L EGTA and 10 µmol/L SNP. For Rho-kinase inhibition, basilar arteries were pre-incubated with Y-27632 (1 µmol/L) for 30 min and ACh relaxation was conducted. Contractile response to angiotensin II (Ang-II) (0.01–3 nmol/L), phenylephrine (PE) (0.001–100 µmol/L), ET-1 (0.01–100 nmol/L), serotonin (5-HT) (0.001–10 µmol/L) and to U46619 (0.01–100 nmol/L) was determined.

Cell Culture and Western Blotting: Mouse aortic smooth muscle cells (MASMC) were isolated from thoracic aorta from S-CUL3Δ9 transgenic mice as previously described (6). MASMC were maintained in high-glucose DMEM supplemented with 10% FBS, 1% glutamine, and 1% penicillin and streptomycin at 37 °C in a 5% CO_2 incubator. Cells were infected with adenoviral Cre to express Cre recombinase driving activation of the transgene. Primary human aortic smooth muscle cells were obtained from ATCC (catalog # PCS-100-012) and maintained as required by the manufacturer. Cells were lysed in radioimmune precipitation assay (RIPA) buffer (1%Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS in 1X PBS with proteinase inhibitors (Roche). Total protein lysates were collected and stored at -80^{o}C until further analysis. Aortas from NT and S-CUL3Δ9 transgenic mice were cleaned of adventitia fat, frozen in liquid nitrogen, and homogenized in RIPA buffer containing protease inhibitors. Total protein lysates were collected and stored at -80^{o}C until further analysis. Equal amounts of protein lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (GE healthcare).

Membranes were blocked with 5% milk and incubated at 4^{o}C overnight with primary antibodies against cullin 3 (catalog # A301-109A, Bethyl), tdTomato (catalog # GTX127897, Gene Tex), GAPDH (catalog # sc-32233, Santa Cruz Biotechnology), cyclin E (catalog # sc-247, Santa Cruz Biotechnology), RhoA (catalog # 2117, Cell Signaling Technology), eNOS (catalog # 9572, Cell Signaling Technology), phospho-eNOS (catalog # 9571, Cell Signaling Technology), phospho-MYPT1 (catalog # 5163, Cell Signaling Technology), β-Actin (catalog # ab16039, Abcam). Immunoblots were then incubated with a secondary antibody conjugated to horse radish peroxidase, and developed using ECL (Amersham Biosciences). Protein bands were quantified using ImageJ NIH software.

RhoA GTPase Pull Down Assay. Active RhoA (RhoA-GTP) was measured using the Active Rho Detection Kit according to the manufacturer's recommendations (catalog # 8820, Cell Signaling Technology). Briefly, whole aortas from NT and S-CUL3Δ9 transgenic mice were cleaned of adventitia fat, cut into 4-5 mm sections and suspended in an organ bath containing Krebs buffer with resting tension set at 0.5 g. Aortas were equilibrated for 30 min and incubated for 5 min with 5-HT (1 μmol/L) or saline. Aortas were quickly removed from the organ bath and frozen in liquid nitrogen. All aortic tissues were homogenized using lysis buffer provided by the manufacturer, and equal amount of protein lysates were incubated with agarose beads containing the Rhotekin binding domain to pull down RhoA-GTP. The agarose beads were collected, boiled for 5 min and proteins separated by SDS-PAGE and analyzed by Western blotting.

Aortic Compliance and Collagen Measurement. Pulse wave velocity (PWV) was measured as previously described (7) using Doppler ultrasound (MouseDoppler^{TM}, Indus Instruments, Texas). Briefly, mice were maintained under anesthesia (2% isoflurane/1% oxygen) in a supine position. Continuous ECG recordings was collected from the paws, and pressure waveforms using a 20 MHz probe were imaged at the descending aorta and abdominal aorta 1 mm above the exit to left renal artery. Arrival and transit times were calculated over 5-7 cardiac cycles, and the distance between the descending and abdominal aorta was measured using a caliper. PWV (m/s) was calculated as distance (m) divided by the time (s). Stress-strain studies were conducted as previously described (8). Briefly, thoracic aortas were dissected free of adventitia fat and cannulated onto steel cannulas in an organ bath containing calcium-free buffer. Using DMT software, intraluminal pressure was increased at 25 mm Hg increments, from 0 to 200 mm Hg and inner and outer diameter, and wall thickness was recorded. Stress–strain relationships where a leftward shift is indicative of arterial stiffness was determined. Adventitial collagen was stained and visualized using Masson's trichrome staining and quantified by planimetry using ImageJ NIH software. As a direct biochemical measurement of total aortic collagen deposition, hydroxyproline content was determined using a biochemical assay as previously described (9).

Drugs and Reagents. ACh, SNP, Ang 1-7, DEA NONO, KCl, cromakalim, nifedipine, serotonin (5-HT), Nω-Nitro-L-arginine methyl ester (L-NAME), Ang-II, and PE were obtained from Sigma; and all were dissolved in saline. $PGF_{2\alpha}$ was from Pfizer; ET-1 was from Peninsula Laboratories and dissolved in water. CN03 was from Cytoskeleton Inc; Y-27632 from Calbiochem, and U46619 was from Cayman Chemical. MLN4924 was obtained from ActiveBiochem and dissolved in DMSO.

Statistical Analysis. All data are expressed as mean ± SEM. Data were analyzed using unpairedpaired, two tailed Student's t-test, one- or two-way ANOVA (repeated measures when appropriate) using Tukey or Bonferroni post hoc tests. $P \leq 0.05$ was considered statistically significant. Data were analyzed using Graphpad Prism (Graphpad Prism 6 or 7 Software).

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